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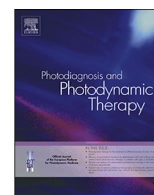
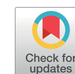


Newly formulated 5% 5-aminolevulinic acid photodynamic therapy on *Candida albicans*



Giuseppe Greco[a,*], Simone Di Piazza[a], Jiemei Chan[b], Mirca Zotti[a], Reem Hanna[b,c], Ezio Gheno[b,d], Angelina O. Zekiy[e], Claudio Pasquale[b], Nicola De Angelis[b,f], Andrea Amaroli[b,e,**]

[a] *Laboratory of Mycology, Department of Earth, Environmental and Life Sciences, University of Genoa, Genoa, Italy*
[b] *Laser Therapy Centre, Department of Surgical and Diagnostic Sciences, University of Genoa, Genoa, Italy*
[c] *Department of Oral Surgery, Dental Institute, King's College Hospital NHS Foundation Trust, Denmark Hill, London, SE5 9RS, UK*
[d] *Dental Clinical Research Center, Dentistry School, Fluminense Federal University, Rua São Paulo, 28, Campus do Valonguinho Centro, Niterói, RJ, 24020 150, Brazil*
[e] *Department of Orthopedic Dentistry, Sechenov First Moscow State Medical University, Trubetzkaya St., 8, Bd. 2, 119991, Moscow, Russian Federation*
[f] *Faculty of Dentistry, University of Technologi MARA, Sungai Buloh Campus, Jalan Hospital, 47000 Sungai Buloh, Selangor, Malaysia*

A R T I C L E I N F O

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A B S T R A C T

Background: A large number of systemic diseases can be linked to oral candida pathogenicity. The global trend of invasive candidiasis has increased progressively and is often accentuated by increasing *Candida albicans* resistance to the most common antifungal medications. Photodynamic therapy (PDT) is a promising therapeutic approach for oral microbial infections. A new formulation of 5-aminolevulinic acid (5%ALA) in a thermosetting gel (t) (5%ALA-PTt) was patented and recently has become available on the market. However, its antimicrobial properties, whether mediated or not by PDT, are not yet known. In this work we characterised them.
Methods: We isolated a strain of *C. albicans* from plaques on the oral mucus membrane of an infected patient. Colonies of this strain were exposed for 1 24 h, to 5%ALA-PTt, 5%ALA-PTt buffered to pH 6.5 (the pH of the oral mucosa) (5%ALA-PTtb) or not exposed (control). The 1 h-exposed samples were also irradiated at a wavelength of 630 nm with 0.14 watts (W) and 0.37 W/cm^2 for 7 min at a distance of < 1 mm.
Results and conclusion: The 5% ALA-PTt preparation was shown to be effective in reducing the growth of biofilm and inoculum of *C. albicans*. This effect seems to be linked to the intrinsic characteristics of 5%ALA-TPt, such acidic pH and the induction of free radical production. This outcome was significantly enhanced by the effect of PDT at relatively short incubation and irradiation times, which resulted in growth inhibition of both treated biofilm and inoculum by ~80% and ~95%, respectively.

1. Introduction

The inclusion of fungi among other commensal organisms of oral biofilms has signified a breakthrough in the knowledge of oral biology [1]. In fact, fungal pathogens can cause a serious health problem for humans; nevertheless, this matter was previously neglected [2]. It was estimated that fungal infections are responsible for in excess of one million human deaths per year [3], attributed to fungi belonging to the genera *Cryptococcus*, *Aspergillus* or *Candida*.

Candida albicans (C.P. Robin) Berkhout 1923 is a yeast-pseudofilamentous dimorphic fungus, which may survive in humans as both a commensal and opportunistic pathogen [4]. It is often responsible for the genesis of invasive candidiasis (IC), which can manifest in several forms, such as candidemia, disseminated candidiasis, endocarditis, meningitis and oral thrush.

The change in status of *C. albicans* from commensal to pathogen form, by its transformation from the yeast state to the pseudohyphoid state, is multifactorial and related to chemical-physical or microbiological variations in the host environment or immunosuppression [5]. Most of the diseases associated with this pathogen were identified by the formation of pseudohyphae biofilms on biotic or abiotic host surfaces [6]. In addition, emerging evidence suggests that there is a link between oral candidiasis and oral precancerous neoplasia [7,8].

A large number of diseases and their treatments, such as diabetes, malignancy, and immune-suppressing therapies, are associated with oral candida pathogenicity. In medically compromised patients, a localized oral infection can spread to the bloodstream, causing a severe systemic infection with an increased rate of morbidity and mortality

* Corresponding author.
** Corresponding author at: Laser Therapy Centre, Department of Surgical and Diagnostic Sciences, University of Genoa, Genoa, Italy.
E-mail addresses: giuseppe.greco@edu.unige.it (G. Greco), andrea.amaroli.71@gmail.com (A. Amaroli).

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[9].

Recently, the global trend in IC has increased progressively at nearly the same rate as fungal infections [10,11]. This tendency is often accentuated by an increase in *C. albicans* resistance to the most common antifungal medications, such as Amphotericin B, Nystatin, Clotrimazole and Fluconazole [12]. Indeed, the mature cells, hyphae and pseudohyphae of *C. albicans* form a three-dimensional structure, which is called an extracellular polymeric substance. This represents an extracellular matrix material, containing β-1,3-glucan, which plays a protective role against the immune system and antifungal medications [13,14]. Notably, IC associated with biofilms are difficult to treat and can contribute to a health risk. Therefore, there is a need to seek alternative therapies which are effective and safe to eradicate *C. albicans* [15].

Photodynamic therapy (PDT) was recently proposed as a promising alternative approach for treating oral biofilms [16] and *C. albicans* [17]. In this respect, 5-aminolevulinic acid (ALA) is known to be an excellent photosensitiser for PDT in the treatment of fungal diseases such as IC [18–20]. Therefore, ALA utilized in an exogenous form has been associated with PDT in several medical fields [21]. Basically, aminolevulinic acid is an endogenous precursor of porphyrins, which can stimulate protoporphyrin IX (PpIX) synthesis in the mitochondria. PpIX is a fundamental molecule in “haeme-group” biosynthesis [22], which features as a chromophore and can be metabolically transformed and accumulated [23]; PpIX is a true photosensitizing agent, which is activated by light and then generates cytotoxic reactive oxygen species and free radicals [24]. Many *in vitro* and *in vivo* dermatological studies showed peculiar characteristics of ALA. Firstly, the small dimensions of ALA permit free access to the stratum corneum of the target cell. Then, the ALA-PDT can be rapidly cleared from tissues [25,26] with shorter-lived photosensitivity [27] and non-cumulative toxicity effects, in comparison to other photosensitizers [2,28]. Lastly, it is also well tolerated by affected oral mucosal tissues, where 20% ALA is suggested in photodynamic therapy guidelines for the management of oral leucoplakia [29].

Nevertheless, a review of the literature shows evidence of a partial reduction in *C. albicans* growth but only when treated with high ALA concentrations and/or high light-energy irradiation, which both could cause a potential risk, even to the cells of the infected host organism, or after long incubation and irradiation times [15,19,23,30].

Lately, a new formulation of 5% ALA in thermosetting gel (t) (5% ALA-PTt) has been patented and placed on the market under the name ALADENT. However, its antimicrobial properties, mediated or not by PDT, are not yet known. Therefore, in this work we characterised them. For this purpose, biofilms and inoculums of *C. albicans* were isolated from plaques on the oral mucus membrane of an infected patient and characterized by morphological and molecular assays. Then, *C. albicans* was exposed for increasing times from 1 h to 24 h to 5% ALA-PTt or 5% ALA-PTt buffered to the mucosal oral pH of 6.5 (5% ALA-PTtb). The sample exposed for 1 h to the drugs was also irradiated for 7 min by LED light at a wavelength of 630 nm, with 58.8 W s, 0.37 W/cm^2, and 154.7 J/cm^2. The capacity of *C. albicans* to grow and to form new biofilms after light irradiation or not, were tested.

2. Material and methods

2.1. Candida albicans isolation and growth

A vital fungal strain of *C. albicans* isolated from a swab taken from plaques on the oral mucus membrane of an infected patient. Subsequently, it was inoculated into Petri dishes containing a modified sterile Sabouraud agar (SAB) media (pH = 4) supplemented with chloramphenicol (C) (40 mg/L). The inoculated Petri plates were placed in a growth chamber at 37 ± 1 °C in a dark environment for 48 h. Then, a vital fungal strain isolated from the plates by repetitive culturing and preserved in axenic cultures, using test tubes with SAB + C medium. Moreover, the isolated microfungal strain was stored at + 4 °C and cryopreserved at –20 °C and 80 °C, in the Mycological collection of Genoa University (DISTAV) for future research. The identity of a yeast-like fungus was confirmed *via* DNA extraction (CTAB method Doyle and Doyle, 1987), PCR amplification of the ITS region (universal primers ITS1F/ITS4 Gardes and Bruns, 1993) and DNA sequencing at Macrogen Inc (Seoul, Republic of Korea). Sequence assembly and editing were performed by using Sequencher® version 5.4.6 (sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA). Taxonomic assignment of the sequenced samples was carried out, using the BLASTN algorithm, in order to compare the sequences obtained in the present study with those in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank).

2.2. Experimental set-up

2.2.1. Five-aminolaevulinic acid (ALA)

In our work, we utilized a newly formulated thermosetting gel of 5% 5-aminolevulinic acid (5% ALA-PTt), labelled as ALADENT Perio & Implant (ALPHA Strumenti, Milano, Italy). The 5% ALA-PTt was employed, either in the unaltered state (pH 3.5) or buffered by sodium hydroxide at the oral mucosal pH of 6.5 (5% ALA-PTtb). The product shows a high level of purity and gels at temperatures above 28 °C; at lower temperatures, ALA is a liquid.

2.2.2. Treatment of C. albicans biofilm with 5-aminolevulic acid

Table 1(A, B) shows a schematic representation of the experimental setup. *Candida albicans* was inoculated on a SAB plate in a Petri dish and incubated in a moist chamber at 37 °C until the growth of a uniform biofilm materialized. A 0.7-cm-diameter biofilm on SAB medium was collected *via* a sterile corer and the core transferred to an empty sterile Petri dish. The core of biofilm was treated with 50 µl of 5% ALA-PTt, 5% ALA-PTtb or untreated (control). Then, the biofilm was incubated in a dark, moist chamber at 37 °C for 1, 3, 6, 9, 12 or 24 h. The sample was washed twice with 2 ml of sterile water to eliminate unmetabolized ALA, where present. The washed biofilm was inoculated on SAB medium in a Petri dish and placed in a dark, moist chamber at 37 °C to grow. The sample was monitored every 24 h for two days (total 48 h), as described in the “Image analysis” section. All the procedures in the presence of ALA were performed in the presence of a very low light intensity, excluding those performed in a dark chamber.

2.2.3. Treatment of C. albicans inoculums with 5-aminolevulic acid

The effect of ALA on the inoculums was assayed, as they have lower cell concentrations and thinner colonies in comparison with biofilms Table 1(C, D) shows a schematic representation of the experimental setup. *Candida albicans* was inoculated in Petri dishes containing SAB medium and incubated in a moist chamber at 37 °C until the growth of a uniform biofilm. A sample of the biofilm was inoculated on SAB medium and treated with 50 µl of 5% ALA-PTt, 5% ALA-PTtb or untreated (control). Then, the inoculum was incubated in a dark, moist chamber at 37 °C for 1, 3 or 24 h. The sample was washed and re-inoculated on SAB medium in a Petri dish and grown in a dark, moist chamber at 37 °C. The inoculum was monitored every 24 h for two days (total 48 h), as described in the "Image analysis" section. All the procedures in the presence of ALA were performed under low light intensity, excluding those performed in a dark chamber.

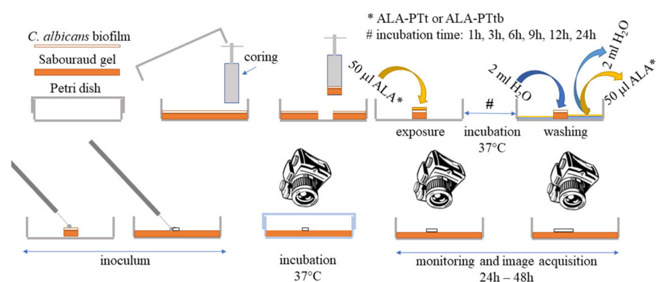
2.2.4. Treatment of C. albicans biofilms and inoculums with photodynamic therapy

The biofilms and inoculums were treated with 5% ALA-PTt or 5% ALA-PTtb for 1 h and incubated as described above (Table 1A, C). Samples that were unexposed to ALA were considered as controls. The samples (treated and control) were washed twice with 2 ml of sterile water to eliminate the unmetabolized ALA, where present. In order to assay the effect of PDT, all the samples were irradiated (Table 1B, D) by

Table 1
Experimental setup to test both the 5-aminolevulinic acid (ALA) and the photodynamic therapy on biofilms (A, B) and inoculum (C, D) of *Candida albicans*. (A, C) Exposure to ALA and monitoring. (B, D) irradiation of sample exposed for 1 h to ALA and washed out. The sample inoculated and its growth monitored and acquired (A, C).

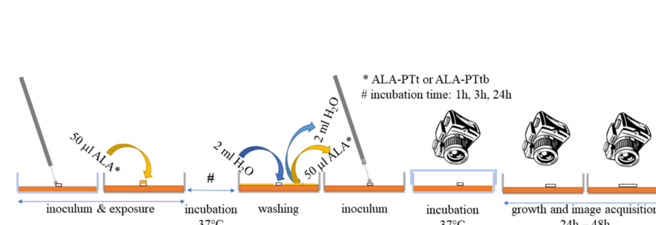
photosensitizer	thermosetting-gel 5% 5-aminolevulinic acid (5% ALA-PTt) 5% ALA-PTt buffered to pH 6.5 (5% ALA-PTtb)
wavelength	630 nm
circle spot area	0.38 cm^2
time irradiation	7 min
power	0.14 W (± 10%)
power density	0.37 W/cm^2
fluence	154.7 J/cm^2

A



B

C



D

an activating LED light source with a wavelength of 630 nm (0.38 cm^2 spot area) and 0.14 W (0.37 W/cm^2, 154.7 J/cm^2) (ALPHA Strumenti, Milano, Italy) at a distance of < 1 mm (~ contact mode) for 7 min [31,32]. After irradiation our biofilms and inoculums were re-inoculated on SAB plates in Petri dishes and placed in a dark, moist chamber at 37 °C to grow. Samples were monitored every 24 h for two days (total 48 h). All the procedures in the presence of ALA were performed in a very-low-light environment, excluding those performed in a dark chamber. According to the manufacturer's instructions, both the incorporation and metabolization of ALA were identified by using the SP 405-N diagnostic fluorescence illuminator (ALPHA Strumenti, Milano, Italy) (emission 365/405 nm; light source wavelength 395 nm). The emission was detected in a dark room and the result was only qualitative (yes/no), not quantitative.

2.3. Image analysis

Both the treated and the control inoculums and re-inoculums were monitored and photographed, by using a Leica MS5 stereoscopic microscope (Leica Microsystems srl, Milan, Italy), which was equipped with a CellPad E camera (TiEsseLab S.r.l.; Italy). The images were obtained *via* fixed magnification and focused parameters. The inoculum areas were measured by three operators with the Image J free software (http://imagej.nih.gov/ij/).

2.4. Statistical analysis

Statistical analysis was performed by using a one-way ANOVA followed by the Tukey-Kramer multi-comparison test (GraphPad InStat 3) to discriminate statistically significant results. The significance levels were defined as follows: high significance level: $P < 0.001$ (*), significance level: $P < 0.01$ (+), significant level: $P < 0.05$ (#), insignificance level: $P > 0.05$. Each experiment was carried out in 3 replications and 10 repetitions.

3. Results

3.1. Candida albicans sample characterization

The isolated cell sample showed biofilm formation and cell morphology ascribable to the genus *Candida* (Fig. 1). The taxonomic assignment was confirmed *via* the BLASTN algorithm, to compare the ITS sequence (deposited in GenBank with accession number MK530515) obtained in the present study, against the GenBank database ascribing, with 99% identity of our strain to *Candida albicans* (C.P. Robin) Berkhout.

3.2. Effect of 5-aminolevulinic acid on C. albicans biofilm

Figs. 2A and B show the results related to the effect of ALA (ALA-PTt or ALA-PTtb) on *C. albicans* biofilms. At 48 h after inoculation, the control samples formed a biofilm with a mean surface area of 0.52 cm^2, significantly greater than that of the initial inoculum.

Samples treated with 5% ALA-PTt for 1 h or with ALA-PTtb from 1 to 24 h showed no effect on the inoculums' growth. However, those samples treated for 24 h and 48 h had a biofilm area that was non-statistically different from the control ($P > 0.05$). Conversely, the effect of 5% ALA-PTt had progressively increased within 3 h of

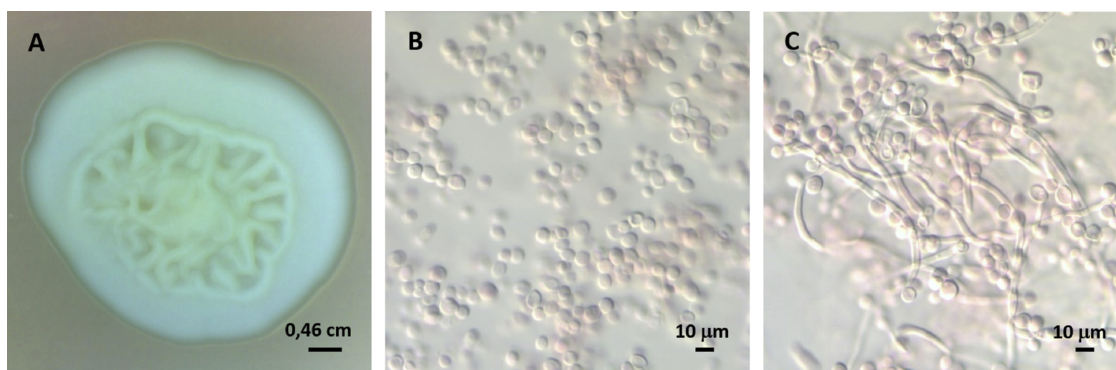


Fig. 1. Shows *Candida albicans* isolated from infected patient and characterised by biomolecular analysis. (A) Biofilm. (B, C) microscopical observation of the biofilm cellular component; (B) yeast stage, (C) pseudohyphae stages on which the experiments were made.

incubation. Moreover, it showed statistically significant differences relative to the control samples ($P < 0.05$). The maximum effect observed at 24 h of incubation was a total inhibition of the inoculum growth ($P < 0.001$).

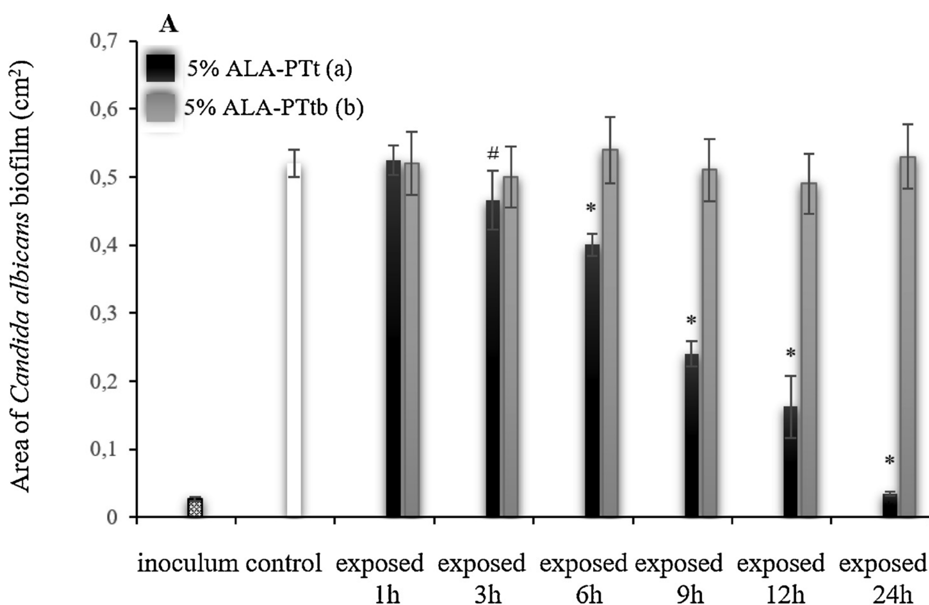
3.3. Effect of 5-aminolevulinic acid on C. albicans inoculum

Fig. 3 shows the results related to the effect of 5% ALA (ALA-PTt or ALA-PTtb) on *C. albicans*. After 24 and 48 h of exposure, the control samples from the inoculum formed a biofilm with an average area of 0.20 (data not shown) and 0.56 cm^2 (Fig. 3), respectively. After 24 h and 48 h, treatment with 5% ALA-PTtb did not block the inoculum growth. However, its area was non-statistically different from that of the control ($P > 0.05$). In contrast, 5% ALA-PTt inhibited *C. albicans* growth after 3 h ($P < 0.01$) and 24 h ($P < 0.001$) treatment exposure periods, but not after the 1 h treatment ($P > 0.05$). Our results, in particular, showed that the 3 h exposure time treatment induced a greater effect ($P < 0.05$) on the inoculum than on the biofilm when exposed to ALA-PTt for the same period.

3.4. Effect of photodynamic therapy on biofilms and inoculums of C. albicans

The samples showed incorporation and metabolization of ALA pointed out by fluorescence emission at 365/405 nm (data not shown).

Fig. 4 shows the results related to the effect of PDT on the samples



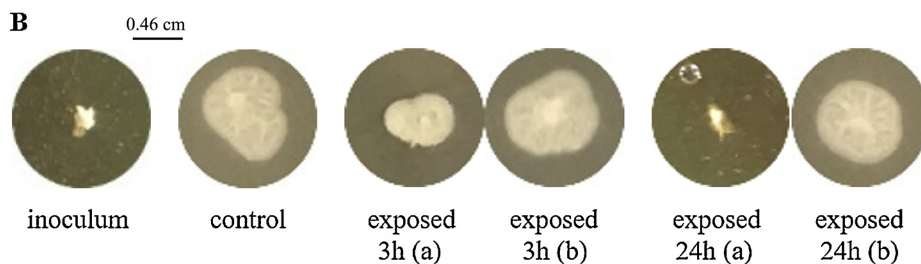


Fig. 2. The effect of 5-aminolevulinic acid (ALA) on *Candida albicans* biofilm growth, 48 h after exposure to drug. The sample were exposed to a thermosetting-gel 5% 5-aminolevulinic acid (5% ALA-PTt) (a), 5% ALA-PTt buffered to pH 6.5 (5% ALA-PTtb) (b) or unexposed (control) for 1 hour (1 h), 3 hours (3 h), 6 hours (6 h), 9 hours (9 h), 12 hours (12 h) or 24 hours (24 h). (A) Averages and standard deviations of the sample area where the inoculum was the area at the time zero. (B) Examples of the biofilm grown 48 h after the treatment. Tukey-Kramer multi-comparison test was performed. The significance was expressed in respect to the control: high significance level: $P < 0.001$ (*), significance level: $P < 0.01$ (+), significant level: $P < 0.05$ (#), no symbols = no significance level ($P > 0.05$).

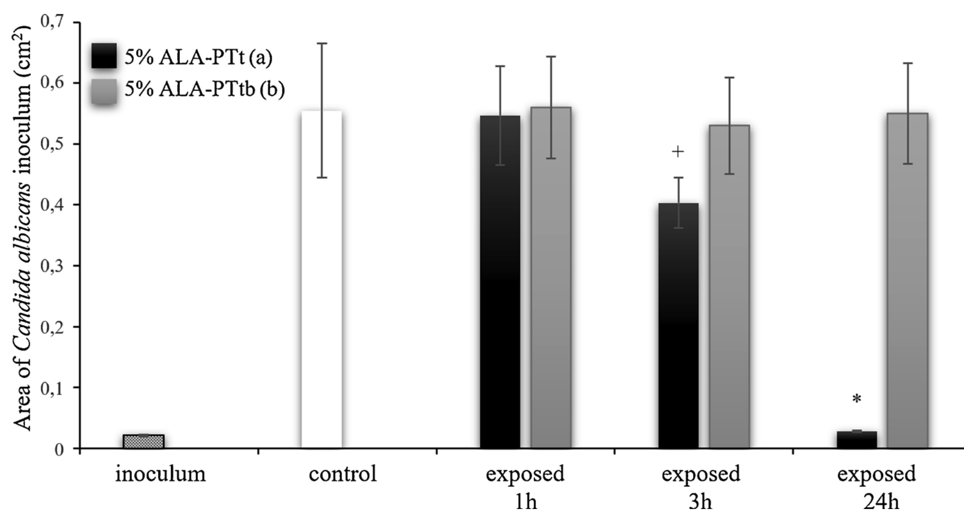


Fig. 3. The effect of 5-aminolevulinic acid (ALA) on *Candida albicans* inoculum growth, 48 h after exposure to drug. The sample were exposed to a thermosetting-gel 5% 5-aminolevulinic acid (5% ALA-PTt), 5% ALA-PTt buffered to pH 6.5 (5% ALA-PTtb) or unexposed (control) for 1 hour (1 h), 3 hours (3 h) or 24 hours (24 h). This figure shows the averages and standard deviations of the sample area, where the inoculum was the area at the time zero. Tukey-Kramer multi-comparison test was performed. The significance was expressed in respect to the control: high significance level: P < 0.001 (*), significance level: P < 0.01 (+), significant level: P < 0.05 (#), no symbols = no significance level (P > 0.05).

incubated with ALA (ALA-PTt or ALA-PTtb) for 1 h and irradiated with 630 nm LED light delivery for 7 min.

The effect of PT on *C. albicans* biofilm treated with ALA-PTt was to inhibit its cellular growth (P < 0.001) by ~80% with respect to the control. However, its effect on the inoculums was greater and reached ~95% inhibition (P < 0.001). Conversely, the results of treating the biofilms and inoculums with ALA-PTtb showed no inhibition of their growth, even though they were irradiated at a wavelength of 630 nm. The results were similar to those of the control (P > 0.05).

4. Discussion

In our work, we evaluated *in vitro* a new formulation of 5% ALA in a thermosetting gel for PDT (5% ALA-PTt) on the biofilm and inoculum of *C. albicans* isolated from an infected patient. Our samples grew on Petri dishes containing a modified sterile Sabouraud agar media and developed a biofilm, indicating the pseudohyphoid stage. Pseudohyphae are reported to be the most aggressive form of the dimorphic *C. albicans* life cycle [33]. Our data showed that 5% ALA-PTt was significantly efficient at slowing down re-inoculated biofilm growth by 10% 70%, in the samples previously treated for 3, 6, 9 and 12 h. In addition, the 1 h exposure treatment was insufficient to influence *C. albicans* growth, but the 24 h exposure to 5% ALA-PTt completely inhibited the ability of *C. albicans* to form new biofilms *via* re-inoculum. Equally, the inoculums exposed to 5% ALA-PTt for 24 h were incapable of forming biofilms in the 48 h after re-inoculation. Moreover, the 3 h exposure period induced greater growth inhibition of the inoculum than of the biofilm. In contrast to the 5% ALA-PTt, the buffered (pH 6.5) ALA (5% ALA-TPtb) has no effect on both *C. albicans* biofilms and inoculums.

It should be noted that an investigation by Uehlinger et al. [34] on the physico-chemical properties of ALA showed that the maximum production of protoporphyrin IX in human cells derived from the lungs and bladder was at physiological pH 7.0 7.6. This indicated the need to buffer the compound containing ALA at this pH level. However, by considering the different cellular features between eukaryotic human cells and the eukaryotic cells of *C. albicans*, the ALA-PTt pH level of 3.5 should have no effect on *C. albicans* survival, because of its ability to tolerate a wide pH range. Indeed, Matsuda et al. [35] data are in agreement that *C. albicans* could survive at acidic pH around a value of 3 and its optimal environment at pH value of 4. In addition, Nadeem et al. [36] evaluated the effect of pH on *C. albicans* growth and demonstrated its resistance to neutral pH (7.4). It is then evident that *C. albicans* is capable of resisting a wide range of pH and expressing its dimorphic fungus-yeast features through a process of changes from the yeast form, which is a typical acidic pH (< 6), to a pseudohyphoid form, which is typical of a neutral-basic pH [36]. Therefore, the effect of ALA-PTt on *C. albicans* after incubations of 3 h or longer is due to the ALA-cell interaction not mediated by the PT.

In this regard, Bechara et al. [37] showed that a precursor of haeme accumulates in various porphyritic disorders (acute intermittent porphyria and tyrosinosis). At high ALA concentrations, its metabolic product induced lipid peroxidation of cardiolipin-rich vesicles, which is

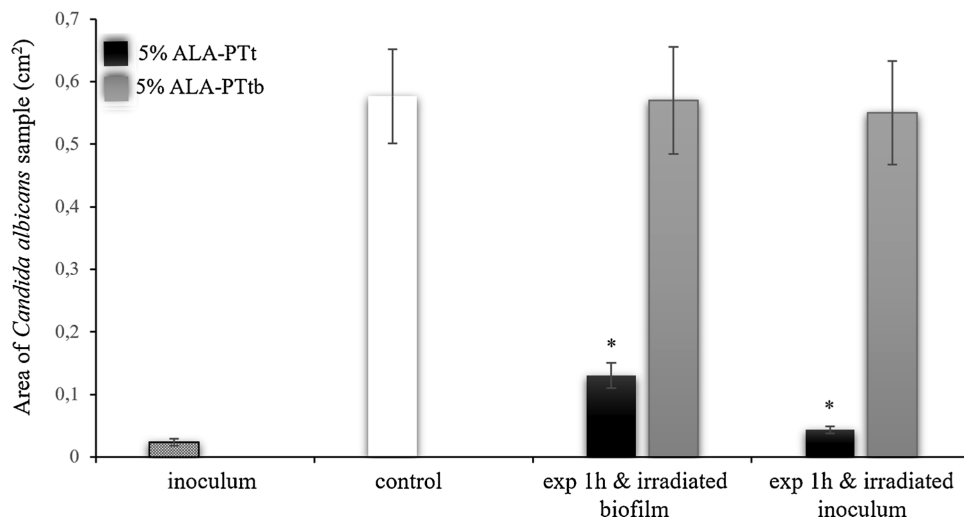


Fig. 4. The effect of photodynamic therapy on *Candida albicans* biofilm and inoculum treated with 5-aminolevulinic acid (ALA), for 48 h after exposure to irradiation. The samples were exposed (exp) to a thermosetting-gel 5% 5-aminolevulinic acid (5% ALA-PTt), 5% ALA-PTt buffered to pH 6.5 (5% ALA-PTtb) (b) or unexposed (control) for 1 hour (1 h). Averages and standard deviations of the area samples of the inoculum was the area at the time zero. The samples were irradiated for 7 min by 630 nm high-energy LED light delivery at power output of 0.14 W. Tukey-Kramer multi-comparison test was performed. The significance was expressed in respect to the control: high significance level: P < 0.001 (*), significance level: P < 0.01 (+), significant level: P < 0.05 (#), no symbols = no significance level (P > 0.05).

a single strand ruptures into a plasmid DNA, as result of ROS production and oxidation of guanosine in calf thymus DNA. Therefore, the effect that we observed in our study would be triggered by the stressful synergistic effect of acidic pH on the pseudohyphae form and by the formation of free radicals induced by high levels of ALA.

Bunke et al. [38] observed that the rate of ALA degradation was inhibited by a pH level < 5 and its rate increased when the pH level increased accordingly. Therefore, the effect observed in our experiments at pH 3.5 (ALA-PTt) but not at pH 6.5 (ALA-PTtb) could be linked to the acidic environment, which kept the ALA metabolically more stable, allowed the preservation of the molecule and sustained it throughout the long incubation time.

Available data on the use of ALA in PDT in treating *Candida* are scanty, and despite the demonstrated reduction in *C. albicans* growth, this occurred after long incubation times and irradiation exposure or at high ALA concentrations. In fact, an *in vitro* study by Monfrecola et al. [30] showed 100% inhibition of *C. albicans* planktonic growth when 630 nm was utilised at a fluence of 40 J/cm^2. However, the ALA-PT concentration in this case was 600 mg/ml (60% ALA) at the 3 h incubation time (50% inhibition by 30% ALA-PT), which exceeds the *in vivo* tolerability level of < 20% ALA-PT [21 and literature cited], which is also suggested for ALA-PT treatment of precancerous oral disease [29].

Shi et al. [15] utilized an ALA-PT concentration much lower than 20%. The results showed inhibition of *C. albicans* growth by 74.5% at a concentration of 15 mM (0.25% ALA) of ALA-PT when irradiated with 635 nm for 5 h. However, applying this protocol *in vivo* is a challenge, due to the lengthy ALA incubation and irradiation exposure periods, at a high fluence of 300 J/cm^2.

Similarly, Oriel and Nitzan [19] noted a reduction from 1.5- up to 2-fold of *C. albicans* planktonic growth when treated with ALA-PT at a concentration of 100 mg/ml (10%) and irradiated with wavelengths ranging from 407 to 420 nm, at a fluence of 36 J/cm^2, for an incubation time of 72 h. This stresses the relevance of our results in terms of a reduction in ALA-TPt concentration to 5% and incubation time to 1 h when it was activated with 630 nm for 7 min (non-thermal irradiation). It is important to clarify that in our experimental set-up we chose a wavelength of 630 nm per the recommendations in the ALADENT Perio & Implant kit and because it is the most often used wavelength in experimental and clinical trials with ALA [32], although blue light (peak wavelength, 456 nm) demonstrated a greater PT effect than red light [31]. This protocol actually demonstrated a near-total inhibition of *C. albicans* growth in one treatment episode on the inoculum and biofilm, which are known to be 2000 times more resistant than the planktonic cells [33]. Finally, it also should be considered that the ALA used in our research is a new formulation that can easily adhere to oral mucosae because of its gel nature at temperatures higher than 28 °C. This should allow a longer ALA incubation time without washout by saliva than the previous liquid formulation; also, if used by injection like in the management of oral leucoplakia [29]. Moreover, an ALA concentration of 5% is well tolerated by human cells compared with concentrations higher than 20% [21] and is less than the 20% ALA suggested in the protocol for treatment of leucoplakia in oral mucosae.

In conclusion, 5% ALA-TPt was effective in inhibiting the growth of C. *albicans*, *in vitro,* on both biofilm and inoculum. This effect seems to be linked to the intrinsic characteristics of 5% ALA-TPt, such as acidic pH and the induction of free radical production. Finally, this outcome was considerably enhanced by the effect of PDT with relatively short of incubation and irradiation times with respect to the ALA therapy generally proposed in the literature. Further *in vivo* investigations are, however, required to verify its feasibility, applications and effectiveness for clinical use.

Ethics approval

Not required.

Author contributions

A.A., M.Z., R.H., E.G, A.O.Z. and N.D.A. conceptualized the study and wrote the manuscript. G.G., S.P., J.C., C.D.P. and A.A. conducted the experiments. G.G., J.C. and A.A. performed the measures for the image analysis. All authors reviewed the manuscript.

Declaration of Competing Interest

All the authors disclose any conflict of interests and no financial supports were obtained for conducting the present investigation.

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